EVALUATION OF SUBSTANCES CAUSING LOSS OF SEBACEOUS GLANDS FROM MOUSE SKIN*†

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Disappearance of sebaceous glands from mouse skin during the first days after exposure to certain carcinogens has been known since 1940 (1). The effect was given significance by Simpson and Cramer (2) who noted that within minutes of painting mice with methylcholanthrene, the carcinogen collected in the sebaceous glands. Simpson and Cramer (3) and Simpson *et al.* (4) further demonstrated that solutions of methylcholanthrene in lanolin which are not carcinogenic to mice fail to destroy sebaceous glands in those animals. They concluded that destruction of the glands may be an early step in the development of skin tumors. Cambel (5) extended their study to include the rat and monkey in which species, methylcholanthrene is not a potent skin carcinogen. The loss of sebaceous glands was not observed even though the hydrocarbon was concentrated in them.

In 1954, Smith *et. al.* (6) reported that the carcinogenic properties of certain petroleum fractions could be predicted by their ability to cause the disappearance of sebaceous glands from mouse skin. Because of the laborious nature of the usual determinations of carcinogenic activity, and the long latent period involved, the use of a rapid test of this type for a screening procedure would be of great value. With this in mind, we undertook to determine what factors might affect the destruction of sebaceous glands by various materials.

The normal numbers of sebaceous glands in a standard section cut perpendicular to the surface of skin varies with the alignment of the tissue with the microtome knife. Furthermore, by this method of cutting only small numbers of glands can be observed in a given section. Suntzeff *et. al.* (7, 8) improved the procedure by cutting skins parallel to the surface. Thus large numbers of glands can be seen in an area rather than a small number in a given length of skin. For our purposes, modification of the Nile Blue staining technic of Lorrain Smith (9) permitted the use of whole mounts of mouse skin. This affords rapid preparation of the tissue while retaining the advantage of study of an entire area.

METHOD

Swiss mice, 55–65 days of age are used. The hair from approximately two-thirds of the back is shaved off with an Oster electric clipper (size 0000 head). On the following three days, each animal is treated, twice daily, with 0.2 ml. of test solution by allowing the liquid to flow from a pipette onto the center of the clipped area.

The mice are sacrificed one week after clipping (four days after the last application) and the treated skin is pinned, fur down, on a dissecting board. Vigorous scraping with the dull edge of a Bard-Parker #12 blade removes the subcutaneous fat. Following scraping,

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the skins are suspended from a cork by means of four pins. They are placed in physiological saline for a period of 3–6 hours, then immersed in 10 per cent formalin overnight.

The fixed tissues are stained in 0.03 per cent aqueous Nile Blue (National Aniline #686) at 68–72°C. for a period of 8 hours. The sebaceous glands and other deposits of neutral fat absorb the oxazone (red) form of the dye, while other structures absorb the ionic oxazine (blue) form. Most of the blue pigment is removed by suspending the tissues in 1 per cent acetic acid for 12 to 16 hours. The tissues are partially cleared in 50 per cent aqueous glycerol for 4 hours, and then in 100 per cent glycerol for 2 to 4 hours. The skins are placed fur side down on a slide and covered, using a drop of glycerol to exclude the air. If necessary, the tissues are lightly scraped again, under glycerin, just before mounting, to remove any remaining subcutaneous debris. They should be examined soon after preparation since noticeable fading occurs within 24 hours.

RESULTS

With the technic described above, all of the sebaceous glands of a skin specimen can be visualized. They appear bright red, generally in pairs and arranged in rough rows, all in the same plane. Other structures are blue. In normal skin of 55–65 day old Swiss mice, the ratio of sebaceous glands to hair follicles is very nearly one to one. When treated with high doses of "sebaceous gland suppressors," the glands are lost completely whereas with smaller doses, partial loss is observed with remnants of many glands still visible.

Suntzeff (7) has reported that unless the hair of the test area is in the resting stage of development, variable response is observed. With whole mounts prepared as above, growing hair follicles obscure the entire field rendering analysis impossible. Accordingly, only skin in the "resting stage" can be used. Among 55–65 day old Swiss mice from our colony, the dorsal skin is in the resting stage of hair development in about 95% of the animals. Any areas of growing hair are readily apparent in the prepared skins, being characterized by very long follicles usually bent by the scraping procedure. They must be disregarded.

Chase *et. al.* (10) have suggested that the most reliable way to obtain resting phase hair is to pluck all of the dorsal hair from a mouse. After 19–21 days, the

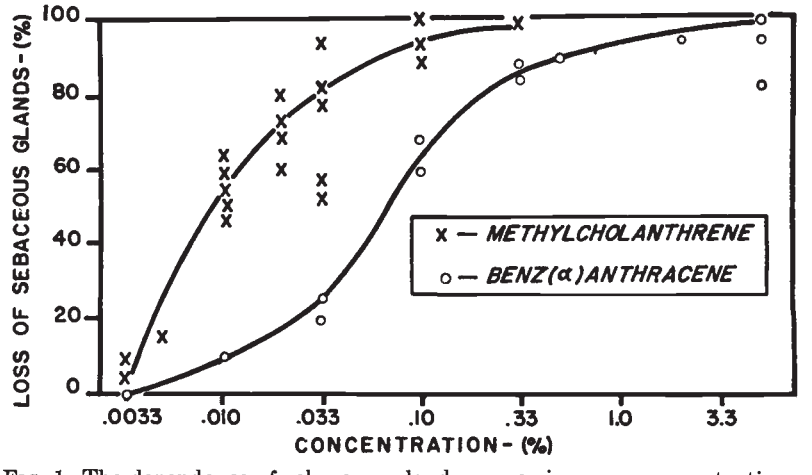

FIG. 1. The dependence of sebaceous gland suppression upon concentration.

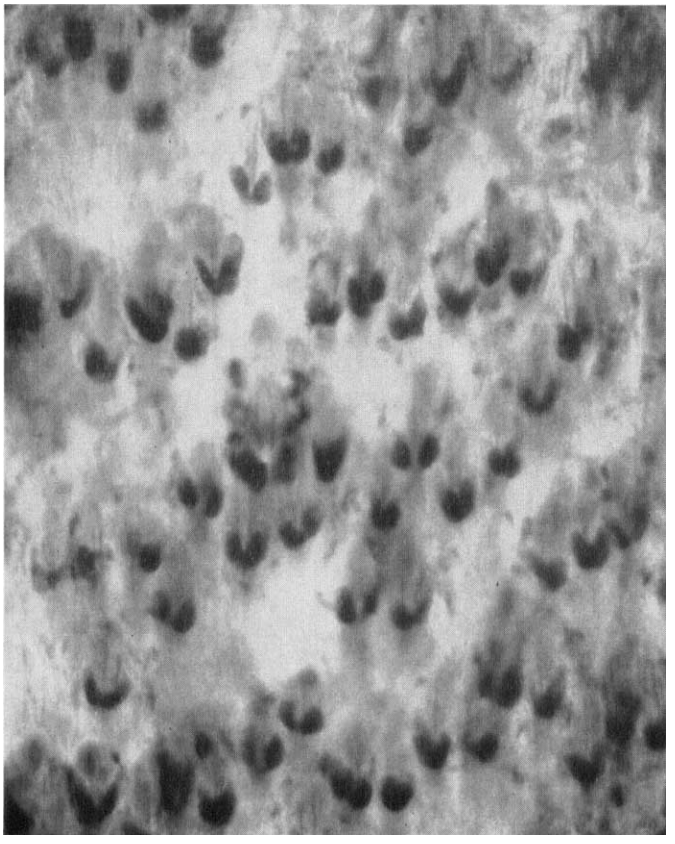

FIG. 2. Normal mouse skin. Upon focusing up and down, it is observed that the hairs are usually grouped in pairs.

new pelage should be entirely in the resting phase. We have found this method to be successful, and of great value when using old mice or animals of doubtful age.

Timing of the painting was designed to permit optimal application of samples that must be dilute because of toxicity or poor solubility. A series of preliminary tests showed that with standard painting, the optimal effect was noted six days after the first application. Painting for the third successive day results in a very slight additive effect.

In order to compare the relative effects of various materials and various methods of application, serial dilutions were employed. Figure 1 demonstrates the dependence of sebaceous gland suppression upon concentration. In all cases, a gland is presumed to be present if any discernible part of it remains. Since changes about the 50% suppression level are most apparent visually, this value is used as an end point. The minimum amount of material that removes at least 50% of the glands from the clipped skin of one test animal under the test conditions is reported as one unit. The sebaceous gland suppressor index is then defined as the number of units of activity per gram of material tested.

FIG. 3. Skin from mouse painted with 0.1% benz(α)anthracene using the standard method.

Two tests were run to determine the effect of time of day and the effect of oxygen tension of the air upon the sebaceous gland suppressive effect of methylcholanthrene. These were prompted by the reports that mitotic activity of mouse skin varies with the time of day (11, 12) and by the possibility that cellular oxygen tension may play a role in carcinogenesis. Since with the standard cages, air circulation is somewhat restricted, the latter factor seemed particularly pertinent.

Table I shows the results obtained from two series of mice painted just once at different times of day. The animals were sacrificed at the same time 6 days after the single painting. Within the limitations of the test, we were unable to detect a significant difference due to this variable.

Table II demonstrates the effect of atmospheric oxygen upon the sebaceous gland suppressor activity of methylcholanthrene. The animals were painted a single time and placed in perforated lucite cages within large lucite chambers, through which streams of air of fixed oxygen concentration were passed. Control animals were left in standard metal cages in room air. It has been demonstrated

TABLE I

The influence of the time of day on "sebaceous gland suppression" by methylcholanthrene

Date	Conc.	Time			
		0500	1100	1700	2300
		% Sebaceous glands destroyed			
	%				
6/27-6/28/55	.18	100	90	100	95
	.06	15	15	20	15
	.02	25	30	10	15
10/5-10/6/55	.10	50	75	65	50
	.05	35	10	15	20

TABLE II

Effect of atmospheric oxygen upon suppressive activity of methylcholanthrene

	Methylcholanthrene Concentration (%)			
	0.2	0.1	.075	.05
	% glands destroyed			
14% oxygen	100	70	45	30
60% oxygen	100	80	35	35
Compressed air	—	50	35	—
Room air	100	50	30	—

by Urbach (13) that the tissue oxygen of mice in such chambers responds to the atmospheric oxygen level within minutes. No significant change could be ascribed to either high or low atmospheric oxygen levels.

Table III lists a series of six compounds having sebaceous gland suppressor activity. With the expection of chrysene, all of these were used as supplied by Distillation Products Industries. The chrysene was obtained from Matheson, Coleman, and Bell. A very small amount of yellow impurity could be removed by washing with hot benzene. The "purified" chrysene had a m.p. of 255°C and possessed an absorption spectrum identical to that reported by Friedel and Orchin (14). It will be noted from the table that the relative sebaceous gland suppressor index is somewhat parallel to the reported carcinogenic activity when measured against mouse skin. It should be pointed out however, that both benzanthracene and chrysene are at best very weak skin carcinogens for the mouse, and that the few isolated reports of skin tumors due to these materials are not consistently confirmed. Consequently, it would appear that qualitative sebaceous gland suppression is also a property of compounds that are at most, very weak skin carcinogens.

Table IV lists a number of compounds which have been tested for sebaceous gland suppressor activity and have been found to be inactive. Many of these compounds deserve special mention. Several are tumorigenic in the mouse for

TABLE III

Sebaceous gland suppressors

Compound	Suppression Index
3-methylcholanthrene	10,000
7,12-dimethylbenz (α) anthracene	10,000
Dibenz (α,h) anthracene	3,000
Benzo (α) pyrene	3,000
Benz (α) anthracene	1,000
Chrysene (impure)	200
Chrysene (purified)	200

The suppression index is defined as the number of units per gram, where the unit is that amount of material that causes the disappearance of at least 50% of the sebaceous glands from the painted area of one mouse.

TABLE IV

Materials that have no demonstrable sebaceous gland supressor effect

Benzene	Squalene	Allyl propionate
Acetone	1,9-benzanthrone	Croton Oil
N-heptane	Phenanthrenequinone	Urethane
Pet Ether 30–60	Quinone	p-dimethylaminoazobenzene
Toluene	1,2-naphthoquinone	2-acetylaminofluorene
Pet Ether 62–69	1,4-naphthoquinone	2-naphthylamine
Ethanol	Hydroquinone	Phenanthrene
N,N-dimethylformamide	Crotonic acid	p-bromophenol
Trioctanoin	Tetrahydronaphthalene	o-iodophenol
Linseed oil	Phenyl acetate	Dinitrophenol
Allyl urea	Anisole	Iodoacetic acid
Linoleic acid	Dodecylbenzene	Pyrene
Octene	Anthraquinone	Anthracene
Oleic acid	Coumarin	Naphthalene

organs other than skin. These include dimethylaminoazobenzene, 2-acetylaminofluorene, and urethane. β-napthylamine is known to be a powerful bladder carcinogen for species other than mice (15).

Benzoquinone (16) and iodoacetic acid (17) have been found to produce skin tumors in mice. Squalene, oleic acid and related olefins were found by Flesch (18) to produce acanthosis in mice after skin painting. Croton oil is a well known co-carcinogen for mouse skin. Since none of the above materials show any activity against the sebaceous glands, it would seem that the latter property is associated with the polycyclic structure of the coal tar carcinogens. The fact that phenanthrene and the keto derivative, 1,9-benzanthrone are inactive lends further support for the evidence that within this class of compounds, sebaceous gland suppression is parallel to carcinogenic activity.

Summation of the suppressive effect is noted in Table V. When five active compounds are combined, the resultant solution is much more active than a solution of any one of them at the same concentration. Sensitivity of the pro-

TABLE V

Summation of sebaceous gland suppression

Compound	% Suppression
.005% Methylcholanthrene (MC)	10
.005 Dimethylbenzanthracene (DMBA)	35
.015 Benzpyrene (BP)	27
.015 Dibenzanthracene (DBA)	40
.05 Benzanthracene (BA)	30
"Combined suppressors" (.005% MC, .005% DMBA, .015% BP, .015% DBA, .05% BA)	70

TABLE VI

Effect of anthracene (A), naphthalene (N), and phenanthrene (P) upon suppressive activity of methylcholanthrene

Sample	% Glands "Destroyed"
.02% MC	75
.02% MC + 1.5% A	70
.02% MC + 10% N	70
.02% MC + 10% P	50
.01% MC	50
.01% MC + 1.5% A	45
.01% MC + 10% N	35
.01% MC + 10% P	30

cedure does not permit determination as to whether the effect is exactly additive, however it does rule out any large degree of either synergism or inhibition between these compounds.

At this time, we have not obtained potent inhibitors of the carcinogenic action of polycyclic hydrocarbons to test against the sebaceous gland effect. That related compounds can inhibit carcinogen activity of coal tar derivatives has been reported by Crabtree (19) and more recently by Hill *et. al.* (20). The preliminary tests which we have made would indicate that neither naphthalene nor anthracene significantly reduce the effect of methylcholanthrene. Phenanthrene seems to have a slight inhibitory effect (Table VI).

DISCUSSION

It is clear that an analysis of the sebaceous gland suppression effect of a sample does not provide an absolute estimate of the carcinogenicity of such material. There are a large number of known carcinogens for both skin and other tissues that do not affect sebaceous glands at all. Furthermore, within the class of polycyclic hydrocarbons related to phenanthrene, compounds that do not cause skin tumors may have weak sebaceous gland suppression activity.

On the other hand within the class of polycyclic hydrocarbons the parallel between ability to produce skin cancer in mice on the one hand and the ability

to suppress sebaceous glands in mouse skin on the other is striking. Because of the ease and reproducibility of this method of assay, it will be of great value under certain circumstances. If a crude sample is known to produce skin tumors in mice and also to cause the loss of sebaceous glands in this species, it would seem reasonable to use the latter test as an assay to guide fractionation. The application of the assay to determine the relative carcinogenicity of various samples seems valid within the limitations imposed by the possibility of inhibition and false positives.

We find that the procedure for skin preparation as outlined above is very rapid and does not require skill in either preparation or analysis. It cannot be used with a high degree of success for thick skinned animals such as the rat. In these animals the tissue cannot be cleared sufficiently to satisfactorily visualize the glands. Recently Hambrick and Blank (21) have described a technic using various agents to free human epidermis with its appendages from the corium. Preliminary trials of their technic lead us to believe that such preparations might be satisfactory for use with rodent skin as well.

SUMMARY

1. A method for preparation of whole mounts of mouse skin for evaluation of effects of "sebaceous gland suppressors" is described.

2. The sebaceous gland suppression effect of methylcholanthrene is not affected by time of painting or oxygen concentration of the air to which the animals are exposed.

3. There is nearly additive summation of the effects of several sebaceous gland suppressors when combined in one solution.

4. Neither naphthalene nor anthracene inhibit the suppression effect of methylcholanthrene, but phenanthrene has a slight inhibitory effect.

5. Among the compounds studied, the sebaceous gland suppression effect is limited to polycyclic hydrocarbons and is parallel to the reported carcinogenic activity of compounds of this group.

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